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Medial pioneer fibers pattern the morphogenesis of early myoblasts derived from the lateral somite

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Abstract

The first wave of myoblasts which constitutes the post-mitotic myotome stems from the medial epithelial somite. Whereas medial pioneers extend throughout the entire mediolateral myotome at cervical and limb levels, at flank regions they are complemented laterally by a population of early myoblasts emerging from the lateral epithelial somite. These myoblasts delaminate underneath the nascent dermomyotome and become post-mitotic. They are Myf5-positive but express MyoD and desmin only a day later while differentiating into fibers. Overexpression of Noggin in the lateral somite triggers their premature differentiation suggesting that lateral plate-BMP4 maintains them in an undifferentiated state. Moreover, directly accelerating their differentiation by MyoD overexpression prior to arrival of medial fibers, generates a severely mispatterned lateral myotome. This is in contrast to medial pioneers that have the capacity for self-organization. Furthermore, inhibiting differentiation of medial pioneers with dominant-negative MyoD also disrupts lateral myoblast patterning and differentiation. Thus, we propose that medial pioneers are needed for proper morphogenesis of the lateral population which is kept as undifferentiated mesenchyme by BMP4 until their arrival. In addition, medial pioneers also organize dermomyotome lip-derived fibers suggesting that they have a general role in patterning myotome development.

Keywords: Avian embryo; BMP; Cell delamination; Desmin; Dermomyotome; Myoblast migration; MyoD; Myf5; Myotome; Pioneer myoblasts; Noggin; Somite

Introduction

The somites develop as epithelial structures in a rostral to caudal gradient along the body axis. Subsequently, they dissociate ventrally to give rise to the mesenchymal sclerotome while the remaining dorsal epithelium becomes the dermomyotome (DM). The sclerotome generates the vertebral column, ribs, tendons, meninges and endothelial cells (reviewed in Christ et al., 2004). The DM is the source of vertebral muscles that form via an intermediate structure, the myotome; it also generates limb muscles, dermis, endothelial cells and cartilage of the scapula blade (reviewed in Scaal and Christ, 2004). The creation of such a complexity stems from a stepwise establishment of a molecular heterogeneity in the young somite and then in the sclerotome and DM, that is translated into specific morphogenetic behaviors and is imposed by local environmental signals (Brent and Tabin, 2002; Buckingham et al., 2003; Hollway and Currie, 2003; Kalcheim and Ben-Yair, 2005; Wolff et al., 2003).

Regarding myotome formation, this complexity is reflected by the stepwise contribution of myoblasts stemming from distinct somite and DM domains. In previous studies, we identified the existence of two distinct waves that account for the formation of the post-mitotic myotome separable by spatial and temporal considerations. A first wave of MyoD and Myf5-positive pioneer myoblasts constitutes the medial region of the epithelial somite; these myoblasts progressively withdraw from the cell cycle, delaminate and migrate towards the rostral somite, and from this region generate myofibers in rostrocaudal and mediolateral directions, gradually filling the somite with unit-length fibers (Cinnamon et al., 2006; Kahane et al., 1998b, 2002). Following the establishment of this primary structure, we showed the advent of a second wave of myoblasts that emanates from all four lips of the DM (Christ

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et al., 1978; Cinnamon et al., 1999; Kahane et al., 1998a, 2002). These results were further confirmed by quail–chick analysis and by lineage tracing with GFP-DNA (Cinnamon et al., 2006; Gros et al., 2004; Huang and Christ, 2000). Further to the formation of a primary, post-mitotic myotome, a third wave of incoming myoblasts was identified, first emanating from the rostral and caudal DM lips (Kahane et al., 2001) and then from the central DM sheet (Ben-Yair et al., 2003; Ben-Yair and Kalcheim, 2005). These progenitors remain transiently proliferative, share a common lineage with dermal cells (Ben-Yair and Kalcheim, 2005) and produce both fibers as well as satellite cells (Gros et al., 2005; Kassar-Duchosoy et al., 2005; Relaix et al., 2005). This precision in the generation of the myotome is likely to result, at least partially, from interactions between myogenic cells of the distinct waves.

Since the growth of the myotome is generally coherent and entails a progressive intercalation between successive waves (Cinnamon et al., 1999; Gros et al., 2004; Kahane et al., 1998a, 2002), we proposed that pioneer myoblasts serve to pattern the cytoarchitecture of the later myogenic waves (Kahane et al., 2002; Kalcheim and Ben-Yair, 2005), but this hypothesis was never experimentally tested in the avian embryo. In the zebrafish somite, elongation of the fast muscle cells depends on a signal generated by the slow muscle fibers that arise medially adjacent to the notochord and migrate from a medial to a lateral position. This outward relocation causes the fast fibers to elongate in a corresponding medial to lateral gradient (Henry and Amacher, 2004). Likewise, in *Drosophila*, founder cells seed the formation of somatic and visceral muscles by associating with fusion competent myoblasts; in the absence of founder cells, fusion-competent myoblasts remain undifferentiated (Baylies et al., 1998; Bour et al., 2000; Ruiz-Gomez et al., 2000).

To begin approaching this question, we first examined the behavior of pioneer fibers at various axial levels and developmental times. The latter was possible due to the rather stable expression of electroporated GFP-DNA over time when compared to previous lineage tracing with lipophilic dyes. Consistent with previous findings (Cinnamon et al., 2006; Kahane et al., 1998b, 2002), we confirm that by 24 h after somite dissociation, at cervical, brachial and flank levels of the axis, pioneer myofibers extend throughout the entire mediolateral myotome that expresses desmin immunoreactivity. A day later, while medial pioneer fibers still attain lateral regions of the myotome at cervical and brachial levels, they reach only about half of the desmin-positive myotome at flank levels of the axis where the hypaxial component is the most significant (see also Gros et al., 2004). Previous studies that addressed the flank region showed that the lateral myotomal component has a relatively late onset becoming apparent about 30 h after the epithelial somite stage and well after the medial myotome had formed (Christ et al., 1983; Cinnamon et al., 1999; Denetclaw and Ordahl, 2000; Gros et al., 2004). These findings raised the questions of the cause for this developmental delay, the precise mechanism that accounts for the initial stages of development of the hypaxial myotome in the flank and its possible relationship with the medial pioneers.

Here we characterize the initial development of the lateral region of the epithelial somite. We find that lateral progenitors withdraw from the cell cycle, delaminate to localize underneath the forming DM as mesenchymal cells yet undergo delayed terminal differentiation in a mediolateral direction concomitant with the arrival of fibers derived from the medial somite. Consistently, these early lateral myoblasts (ELMs) express Myf5 but lack MyoD and desmin expression which contrasts with the medial pioneers that express MyoD and Myf5 when still epithelial. This relative delay in fiber differentiation is due to the antimyogenic effect of somatopleural BMP4, as local treatment with noggin produces fibers prematurely. In addition, we provide evidence that organization of the ELMs into ordered fibers depends upon signals from the medial pioneers arriving to the center of the flank myotome, as overexpression of MyoD which induces the ELMs to prematurely differentiate prior to arrival of the medial fibers, results in a severely disorganized lateral myotome. Reciprocally, abrogating pioneer fiber differentiation with dominant-negative MyoD prevents lateral myotome patterning. Furthermore, we extend our analysis to show that medial pioneers are also responsible for the correct patterning of fibers originating along rostral and caudal DM lips, thus providing the first evidence for a general organizing role of this early myogenic population.

Materials and methods

Embryos

Fertile quail (*Coturnix coturnix japonica*) eggs from commercial sources were used.

Expression vectors and electroporation

Five expression vectors were employed: pCAGGS-AFP, full-length chicken MyoD-pCAGGS (Delfini and Duprez, 2004), a dominant-negative version of MyoD that consisted of the bHLH domain of mouse MyoD fused to the engrailed repressor domain (MyoD(bHLH)-enR); a control plasmid in which the basic region was replaced by the corresponding region of the MyoD's non-myogenic dimerization partner E12 (E12 basic (bHLH)-enR) (Steinbach et al., 1998), and xNoggin (Endo et al., 2003). The latter three were subcloned into the pCAGGS vector.

Electroporations were performed under a dissecting microscope. DNA (1–4 μg/μl) was microinjected into the center of flank-level epithelial somites. Several different types of electroporation were performed, to the medial, lateral, dorsal, and combined rostral/caudal regions of epithelial somites, or to the early-formed DM. Medial and lateral electroporations were performed by inserting sharpened tungsten electrodes into the coelomic cavities on both sides of the embryo. For ventral to dorsal transfections, the negative electrode was inserted under the endoderm and the positive dorsal to the ectoderm, both confined to the somitic area. For combined rostral/caudal transfections, the negative electrode was placed under the endoderm and the positive one dorsal to the intersomitic cleft three somites apart from each other. A four-parameter Pulse*Agile* square wave electroporator (PA-4000, Cyto Pulse Sciences, Inc.) was used. Two protocols differing in intensity were employed, for lineage tracing purposes 3 groups of sequential pulses were delivered as follows: 3×12 V, 20 ms each; 1×24 V, 5 ms; 3×12 V, 20 ms each. For functional assays, 3 groups of stronger pulses were given as follows: 3×30 V, 20 ms each; 1×38 V, 5 ms; 3×30 V, 20 ms each. In some cases, double electroporations were performed, 2 h apart, to

either medial and lateral or medial and rostral/caudal somite domains, respectively. Medial electroporations, aimed at transfecting pioneer myoblasts were of the high voltage type and lateral or rostrocaudal electroporations were of the low voltage type. In all cases, low voltage electroporations were given first. Some electroporated embryos received a pulse of Brdu (10 mM) for 1 h prior to fixation.

Tissue processing, immunocytochemistry and in situ hybridization

Embryos were fixed with 4% formaldehyde in PBS, and either embedded in paraffin wax and sectioned at 6 or 8 μm, or observed as whole-mount preparations following evisceration and flattening. Immunolocalization of p27 was performed on cryosections. Immunostaining for desmin and Brdu was as described (Kahane et al., 2001). Rabbit anti-GFP (Molecular Probes) was used at 1:500 alone, in combination with desmin or Brdu immunolabeling or with in situ hybridization for *MyoD*. Additional hybridizations for *Myf5*, *p27* or *Sim1* were as described (Cinnamon et al., 2001). Nuclei were visualized with Hoechst. Whole-mount embryo preparations and sections were photographed using a DP70 (Olympus) cooled CCD digital camera mounted on a BX51 microscope (Olympus).

Results

The early myotome at flank levels of the axis is composed of medial pioneers and early lateral myoblasts (ELMs)

Axial-level differences in the contribution of medial pioneers to the myotome

Electroporation of GFP-DNA to the medial aspect of epithelial somites produces 24 h later myofibers that span the mediolateral extent of the desmin-positive myotome, and this feature is common to cervical, wing and flank levels of the axis (Figs. 1A–C). This confirms our previous findings in which a population of pioneer post-mitotic epithelial cells of the medial somite expressing MyoD, Myf5 and desmin generates an initial myotome (Cinnamon et al., 2006; Kahane et al., 1998a,b, 2002). Forty-four hour after electroporation, the pioneer fibers still occupy most of the mediolateral domain of the desmin-

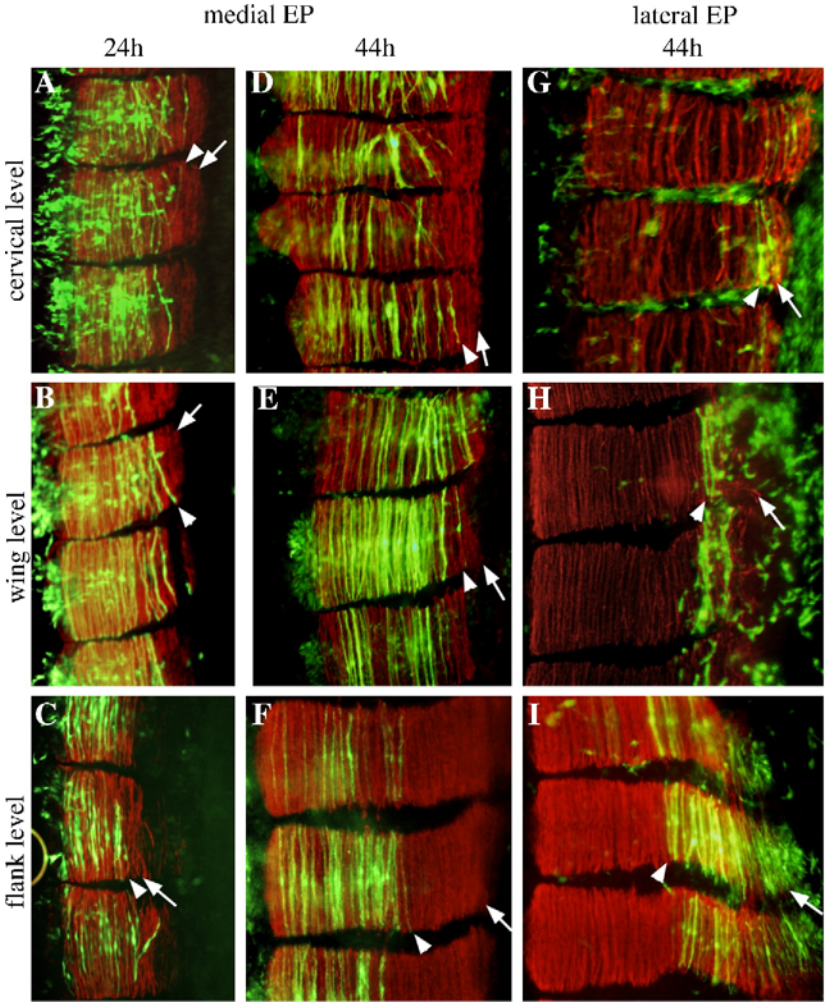

Fig. 1. The establishment of the myotome by medial pioneers is complemented, at flank levels, by lateral somite-derived myoblasts. (A–F) Electroporations (EP) of a GFP-encoding DNA into the medial aspect of epithelial somites at cervical (A, D), wing (B, E), and flank (C, F) levels of the axis fixed at 24 h (A–C) or 44 h (D–F). (G–I) Corresponding electroporations directed to the lateral aspect of epithelial somites fixed at 44 h. (A–C) By 24 h after electroporation, the medial pioneers (green) spread across most of the mediolateral extent of the desmin-positive myotome (red) at all axial levels. (D–F) By 44 h, pioneer fibers arising from the medial somite still attain most of the mediolateral extent of the desmin-positive myotome at cervical (D) and wing (E) levels, but are limited to the medial half of the myotome in the flank (F). (G–I) Electroporation of the lateral epithelial somite reveals in the flank (I), the generation of lateral fibers complementary to the medial pioneers. In contrast, the lateral contribution to cervical and wing levels is very limited (G, H). Arrows and arrowheads mark the lateral limit of the desmin+ myotome and of the GFP+ medial fibers, respectively. Medial is to the left in all panels.

positive myotome at cervical and wing levels (Figs. 1D, E) but remain confined to the medial half in the flank (Fig. 1F). Similar electroporations to the lateral region of epithelial somites revealed that the contribution of this domain to myofibers was very significant in the flank where it seems to complement the medial pioneer fibers (Fig. 1I and Gros et al., 2004), but was very limited at cervical and wing levels (Figs. 1G–I).

Characterization of the initial stages of ELM development relative to medial pioneers

A time course analysis of the development of ELMs was undertaken. Four hours following GFP plasmid transfection, labeled cells were still in the lateral epithelial somite (Fig. 2A). As early as 7 h, corresponding to the dissociating somite stage, a group of GFP+ ELMs had already delaminated underneath the forming DM. More such mesenchymal cells were apparent by 12 h, which still lacked desmin expression (Figs. 2B, C). By this time, the desmin-positive medial pioneers progressively differentiated in a dorsomedial to ventrolateral direction. By 19–20 h, medial fibers encountered ELMs; by this time, the medial myotome accounts for the majority of desmin+ fibers while the first GFP+ ELMs begun differentiating into fibers and coexpressing desmin immunoreactivity (arrowheads in Figs. 2D, G). Notably, the earliest lateral fibers were adjacent to the mediolaterally advancing front of desmin immunoreactivity while ventrolateral ELMs were still mesenchymal and lacked desmin. Hence, acquisition of desmin expression and differentiation into fibers progressed in a general medial to lateral direction, in direct continuity with the gradient of development of the medial myotome (Figs. 2B–H). By 44 h, ELMs already spanned about half of the lateral myotome complementing the medial myofibers (Figs. 1I and 2I).

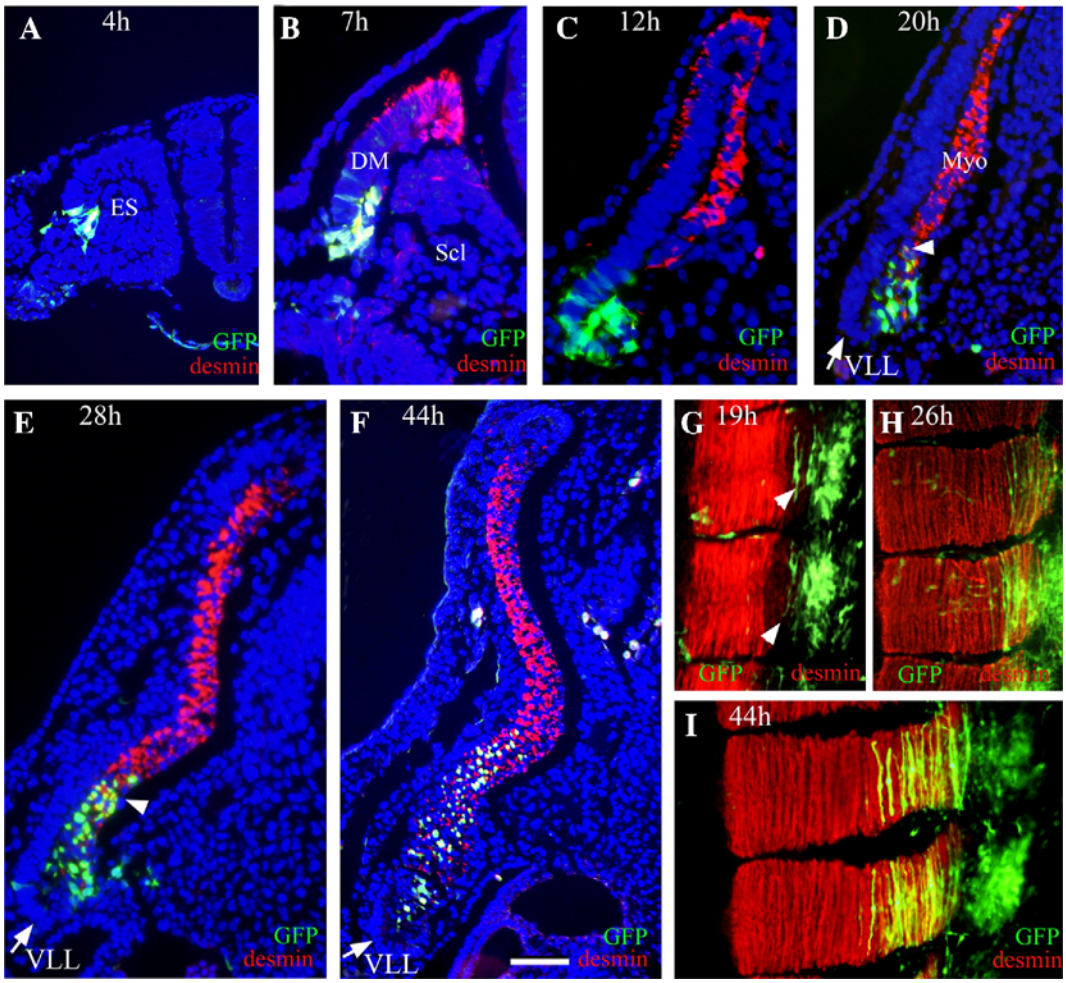

Fig. 2. Time course of the development of early lateral myoblasts (ELMs). ELMs were lineage traced by electroporating the lateral somite with GFP/DNA. (A–F) Transverse sections illustrating the time-dependent development of lateral myofibers vis-à-vis expression of desmin protein. (A) 4 h, (B) 7 h, (C) 12 h, (D) 20 h, (E) 28 h, (F) 44 h. (G–I) Whole-mount preparations showing similar lateral electroporations after 19 h (G), 26 h (H) and 44 h (I). Four hours after electroporation (A), ELMs are still epithelial; by 7 h, they begin delaminating and localize as desmin-negative mesenchymal cells underneath the nascent DM. ELMs continue accumulating until about 20 h (C, D), a time when most ELMs are still mesenchymal and desmin-negative (see also panel G). Note that at these stages, desmin expression identifies the medial pioneers. Between 20 and 28 h, the medial most located ELMs are already desmin-positive and differentiated into fibers (arrowheads in panels D and E, see also panels G and H) whereas the lateral subset of ELMs is still mesenchymal (E, H), showing a medial to lateral order of differentiation. GFP+ ELM differentiation into desmin+ fibers is further accentuated at 44 h (F, I). Note in panels D–F (arrows) that the ventrolateral lip (VLL) of the DM remains GFP-negative demonstrating that the ELM population is a limited cell subset rather than a population arising via a stem-like mechanism. Abbreviations, ES, epithelial somite; DM, dermomyotome, Myo, myotome, Scl, sclerotome. Scale bar = A, B, D, 67 μM; C, 77 μM; E, 50 μM; F, 84 μM.

In previous studies it was suggested that the DM lips are likely to act as stem cell systems that produce both myofibers as well as additional epithelial lip cells (Cinnamon et al., 2006; Gros et al., 2004; Ordahl et al., 2001; Venters and Ordahl, 2002). In this context, it is worth emphasizing that in 65% of cases ($N=40$), the VLL of the DM remained unlabeled following release of the ELMs (Figs. 2D–F, arrows). If a stem cell mechanism operated in the nascent VLL, we would have observed the production of labeled ELMs along with residual labeling of the epithelial VLL in all transfected cases. Hence, similar to medial pioneers (Cinnamon et al., 2006) the ELM population also consists of a limited set of progenitors instead of being a population that arises via a stem-like mechanism from the emerging VLL.

ELMs withdraw from the cell cycle upon dissociation from the somite epithelium

The mitotic ability of ELMs was examined by combining lateral electroporation with a GFP-encoding plasmid and Brdu immunolocalization following a 1-h pulse prior to fixation. GFP+ ELMs were Brdu+ 4 h after transfection when still in the epithelial somite (Figs. 3A, B, arrows). In contrast, medial pioneers already exhibited reduced incorporation of Brdu (asterisks in Figs. 3A, B) as well as high levels of *p27* transcripts and protein (Figs. 3C, D), a bona fide G1/S inhibitor acting at the level of cyclinE/cdk2 and cyclinD/cdk4/6 complexes (Slingerland and Pagano, 2000). Twelve hours following transfection, both medial pioneers as well as ELMs were Brdu-negative and expressed *p27* mRNA and protein (Figs. 3E–H, arrows). Taken together, these data show that ELMs rapidly dissociate to localize underneath the DM and begin exiting the cell cycle, yet they are delayed relative to medial pioneers both in terms of withdrawal from the cell cycle (Fig. 3) and of myogenic differentiation (Fig. 2).

ELMs are Myf5+/MyoD− and acquire MyoD immediately prior to differentiation

The medial region of the epithelial somite that comprises the pioneer myoblasts already expresses *MyoD* and *Myf5* mRNAs (Figs. 4A, B). Myf5 is also expressed faintly throughout the dorsal somitic epithelium and in particular in its lateral domain. In contrast, no MyoD transcripts are detected in the lateral domain (Figs. 4A, B arrows). At this stage, while Myf5+ medial pioneers are mostly Brdu-negative (Fig. 4C, asterisks), Myf5+ cells in the lateral region still actively proliferate (Fig. 4C, arrowheads). During the early phase of DM development (10–15 h after somite dissociation), when *MyoD+/Myf5+* pioneers advance ventrolaterally, *Myf5* transcripts become apparent in the mesenchymal ELMs which at this stage are mostly Brdu-negative (Figs. 4E, F), and in the overlying DM, a pattern that persists through later stages (Figs. 4E, H). In contrast, the region containing ELMs is still devoid of *MyoD* (Fig. 4D). *MyoD* is upregulated in the ELMs only at mature DM stages, about 20 h following somite dissociation when their differentiation is underway (Fig. 4G). These data indicate that Myf5 alone is not sufficient for ELM fiber differentiation, and suggest that the later differentiation of ELMs vis-a-vis the medial myotome results from delayed expression of *MyoD*.

Lateral mesoderm-derived BMP4 maintains ELMs in a progenitor state

BMP4 is an antimyogenic factor (Hirsinger et al., 1997; Pourquie et al., 1995, 1996; Reshef et al., 1998). In the mesoderm, a gradient of *BMP4* mRNA expression is apparent

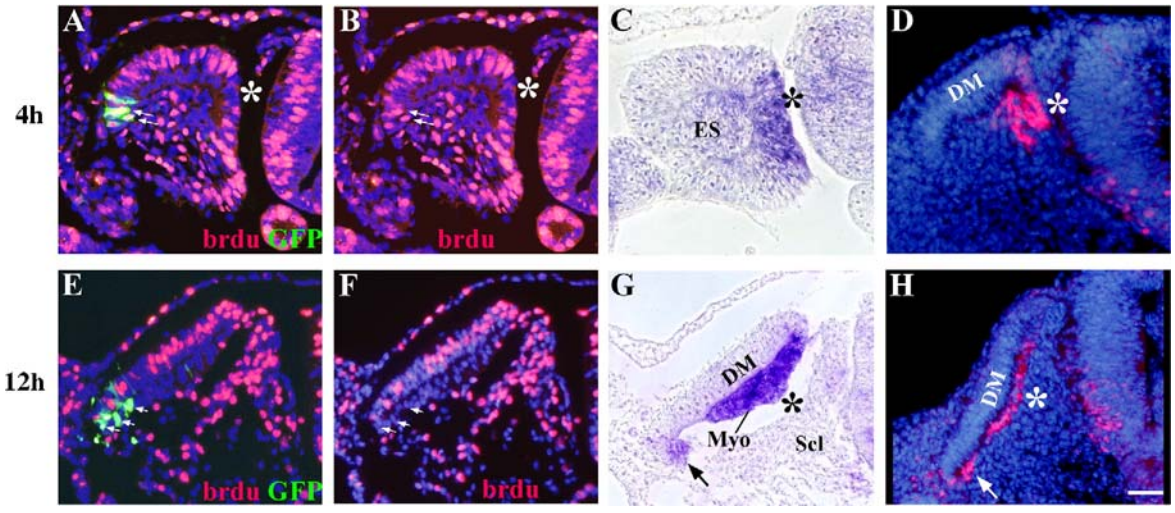

Fig. 3. ELMs exhibit reduced proliferation as early as 8 h following the onset of somite dissociation. The proliferation of ELMs was monitored by combined lateral electroporation of GFP-DNA and incorporation of Brdu following a 1-h pulse prior to fixation. (A, B) Four hours after electroporation, the ELMs (green) are still epithelial and incorporate Brdu (pink, arrows). In comparison, note that the area corresponding to the medial pioneers (*) already exhibits low Brdu incorporation relative to the adjacent epithelium. (C, D) The medial epithelial somite (*) also expresses p27 transcripts (C) and protein (D). (E, F) 12 h after electroporation. A certain number of labeled ELMs reached the underlayer of the DM epithelium and are already Brdu-negative (arrows). Note as well that the VLL region also reveals a reduced extent of Brdu incorporation (Ben-Yair et al., 2003). By this time, the Brdu-negative ELMs are still devoid of desmin immunoreactivity and have a mesenchymal appearance (see Fig. 2C). (G, H) At this time, p27 mRNA (G) and protein (H) are expressed both by medial pioneer fibers as well as by ELMs (* and arrows, respectively). Abbreviations, ES, epithelial somite; DM, dermomyotome, Myo, myotome, Scl, sclerotome. Scale bar=A–D, 30 μM; E–H, 40 μM.

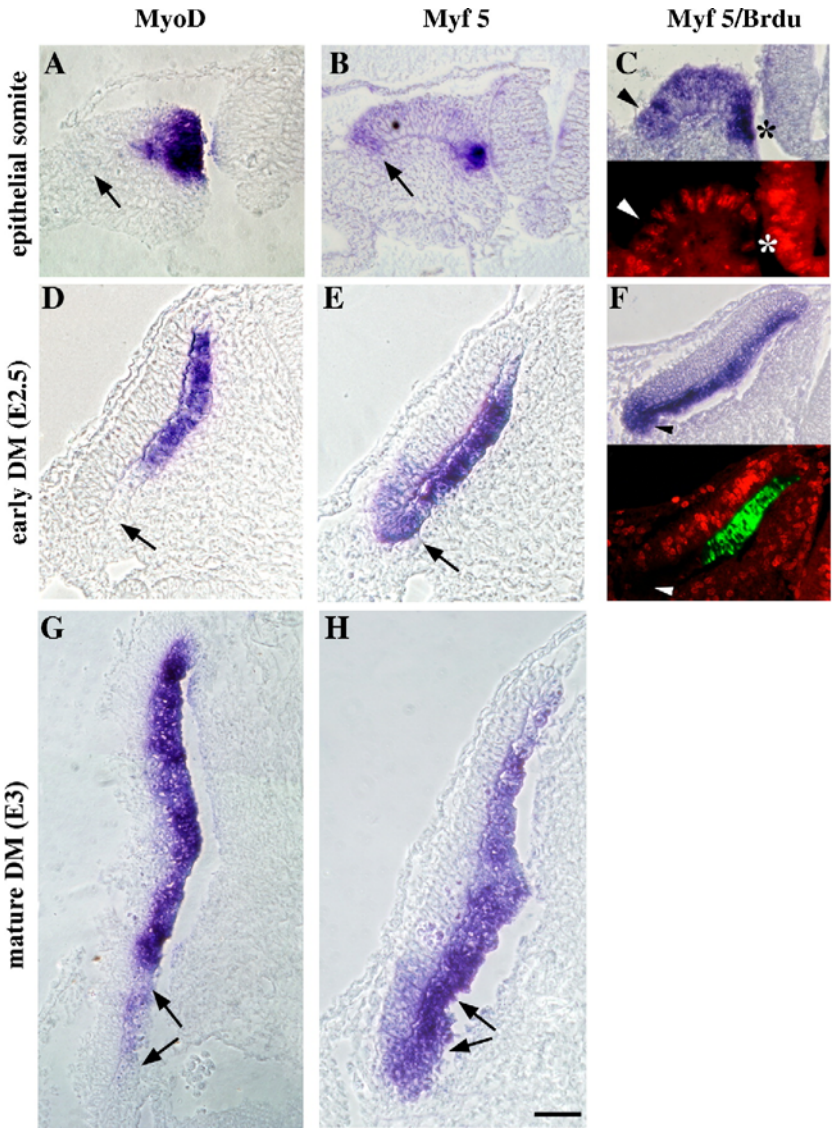

Fig. 4. ELMs express Myf5 mRNA, but not MyoD transcripts. In situ hybridizations for MyoD (A, D, G) and Myf5 (B, C, E, F, H) of sectioned embryos at the epithelial somite stage (E2, A–C), following somite dissociation (E2.5, D–F) and at a mature DM stage (E3, G, H). (A, B) Both MyoD and Myf5 are enriched in the medial aspect of the somite. Note that MyoD mRNA is absent from the lateral somite region both at the epithelial and early DM stages and even at E3 it exhibits weak expression compared to more medial regions (arrows). In contrast, Myf5 transcripts are present at all stages in the lateral domains of both DM and nascent myotome (arrows). Whereas Myf5+ medial epithelial pioneers are devoid of Brdu labeling, Myf5 + ELMs are still Brdu+ at the epithelial somite stage (C, upper panel Myf5 and lower panel Brdu, * depicts medial pioneers and arrowheads the ELMs). By E2.5, Myf5+ ELMs are already Brdu-negative when compared to the more medial region of the DM (arrowheads in panel F, upper and lower panels as in panel C). Scale bar = 40 μM.

in both the intermediate and lateral plate mesoderm (LPM) where it is intensely transcribed adjacent to the segmental plate, comparatively weaker facing epithelial somites and very faint or undetectable in the LPM juxtaposed to dissociated somites (Sela-Donenfeld and Kalcheim, 2002). Since the observed downregulation corresponds to levels where ELMs begin developing (Figs. 2–4), we tested the hypothesis that the activity of lateral BMP4 accounts for the observed delay in ELM differentiation. To this end, the specific BMP4 inhibitor noggin was co-electroporated along with GFP into lateral epithelial somites. Twenty hours later, the presence of enlarged myotomes was observed in all transfected segments, with a significant lateral component consisting of slightly mispatterned desmin/GFP-positive fibers. In contrast, the contralateral myotomes were narrower, with a majority of fibers being at this stage of medial origin ($N = 9$, Figs. 5A–F and see also Fig. 2D). Notably, the enlargement observed was associated with an upregulation of *MyoD* in the ELMs when compared to contralateral sides that were still devoid of this gene ($N = 9$, Figs. 5G–I). These data suggest that BMP4, through inhibition of *MyoD* expression, is responsible for transiently maintaining ELMs in a progenitor state, thus preventing premature fiber differentiation.

Inducing premature differentiation of ELMs prior to medial myotome arrival, results in abnormal fiber patterning

To begin understanding whether the relative delay in fiber differentiation of ELMs compared to medial pioneers is of

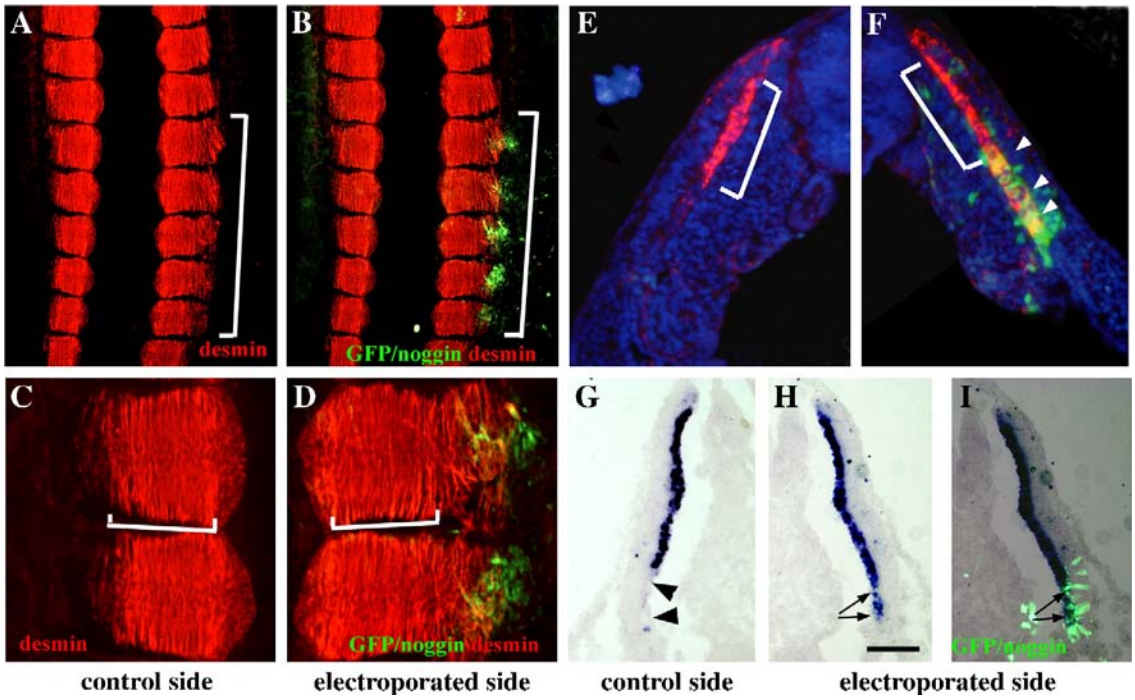

Fig. 5. Lateral plate-derived BMP4 maintains ELMs in a progenitor state. The lateral domain of flank-level epithelial somites were co-electroporated with noggin and GFP-encoding DNAs. Twenty hours later, embryos were fixed and immunostained for desmin (red) and GFP (green). (A–F) Note in panels A and B that the desmin-positive myotome is enlarged in electroporated segments (delimited by a white line) when compared to the contralateral intact side. The enlargement in panel D of the electroporated side better reveals that mesenchymal ELMs prematurely differentiated into fibers localized lateral to the white brackets when compared to the control side (C). (E, F) Transverse section of a similar embryo showing in panel F the differentiation of the treated ventrolateral myotome that coexpresses both GFP and desmin (arrowheads) when compared to the control side (E). White lines in panels E and F delimit the extent of the control myotome. This embryo was eviscerated prior to sectioning. (G–I) A similar embryo that received a lateral electroporation of noggin/GFP and was in situ hybridized for MyoD (blue) and co-stained for GFP. (G) Control side, (H, I) treated side with panel I representing an overlay of both MyoD and GFP. Consistent with precocious fiber differentiation in noggin-treated lateral somites, a premature upregulation of MyoD mRNA is also visible in the lateral region of the electroporated segment (arrows in H and I) when compared to the contralateral side that contains MyoD-negative ELMs (arrowheads in panel G). Scale bar = E, F, 50 μM; G–I, 60 μM.

developmental significance, we induced premature fiber generation by overexpressing MyoD, shown to be associated with fiber differentiation (Figs. 4, 5). Lateral electroporation of control-GFP revealed 20 h later the presence of many mesenchymal ELMs and few labeled fibers expressing low levels of desmin immunoreactivity. A gap was still present at this stage between the medial pioneer fibers expressing desmin and the lateral myoblasts (Fig. 6A and see also Figs. 2D, G). Transfection of MyoD/GFP resulted in massive and premature differentiation of ELMs into myofibers coexpressing GFP and desmin. Nevertheless, these fibers were completely misspatterned and lacked a normal parallel arrangement ($N=8$, Figs. 6B–D). This phenotype was more severe than that of the precocious fibers observed upon noggin overexpression, likely because of the faster effect of MyoD which acts downstream of BMP/noggin. Moreover, this misalignment could still be observed 2 days after electroporation (Figs. 6E, F). In addition, whereas noggin overexpression reduced transcription of the lateral marker *Sim1*, consistent with a role for BMP signaling in mediolateral somite specification (Pourquie et al., 1996), a similar lateral electroporation of MyoD had no effect on *Sim1* expression levels hence dissociating between mediolateral identity and fiber differentiation (Supplementary Fig. 1). These data further imply that MyoD acts downstream of BMP/noggin to promote fiber differentiation without affecting the identity of the hypaxial cells.

In striking contrast, when MyoD/GFP was similarly transfected into the medial somite, a prominent enlargement of the medial myotome was observed when compared to segments that received control-GFP, and fibers were correctly organized along the medial to lateral extent of the myotome ($N=10$, Figs. 6G, H). These results suggest that whereas medial pioneers, the first myogenic population of the somite, have the ability to self-organize, the ELMs require an extrinsic signal/s for proper assembly. Since the first lateral fibers differentiate in apposition to the advancing front of medial pioneers and in continuity with them, and since premature differentiation of ELMs preceding medial fiber arrival resulted in abnormal lateral fibers, we hypothesized that this patterning signal/s emanates from the medial pioneers. To begin testing this possibility, MyoD/GFP was electroporated into the overlying DM sheet, which does not normally express MyoD neither does it generate fibers. Nevertheless, upon MyoD overexpression, DM progenitors rapidly differentiate into fibers that translocate into the underlying myotome. In the myotome, two distinct regions were observed, a medial region where the MyoD+ DM-derived fibers met the preexisting medial pioneers and gave rise to ordered parallel fibers (Figs. 6I–K, area delimited by the

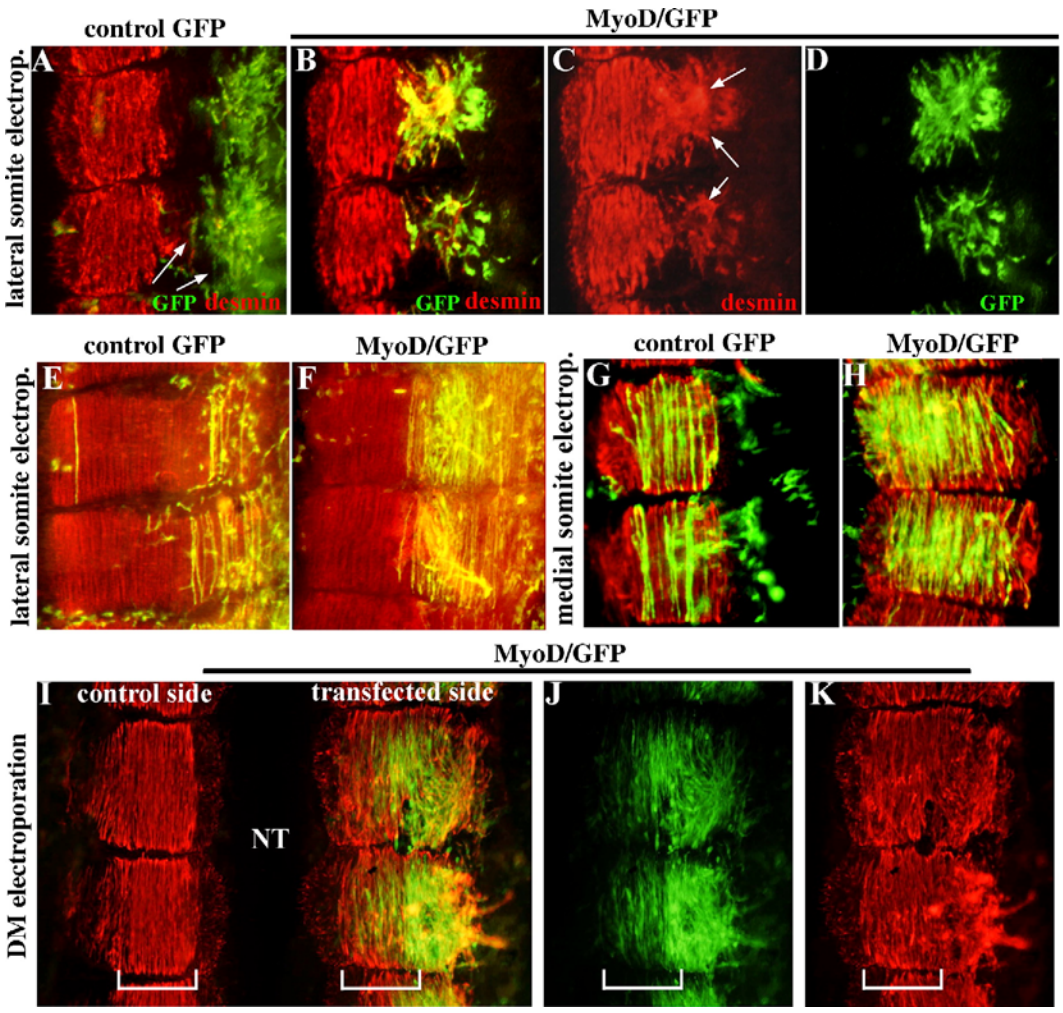

Fig. 6. Premature differentiation of ELMs induced by MyoD prior to arrival of medial pioneers, results in abnormal myofiber patterning. Flank-level epithelial somites (A–H) or DM sheet (I–K) were electroporated with control GFP or MyoD/GFP. (A) Control GFP to the lateral domain of somites shows 20 h later many GFP-labeled mesenchymal cells and the beginning of fiber differentiation exhibiting low desmin expression (arrows). At this time point, there is still a gap between the lateral limit of strong desmin expression reflecting the front of medial pioneers and the location of ELMs. (B–D) Similar electroporation with MyoD/GFP results in massive fiber differentiation which occurs in the absence of adjacently differentiated medial pioneers (note in panel A the gap between the desmin+ domain and the normal localization of ELMs still apparent after 20 h). MyoD-induced fibers express intense desmin immunoreactivity (arrows in panel C). These fibers are highly misspatterned lacking a normal parallel arrangement (C, desmin, D, GFP and B, merge). (E, F) Fiber mispatterning is still apparent 48 h following electroporation (compare panel F with panel E). (G, H) Medial epithelial somites were similarly electroporated with control GFP (G) or MyoD/GFP (H). By 20 h, MyoD both accelerated and enhanced pioneer fiber differentiation while keeping their normal parallel patterning. (I–K) Electroporation of MyoD/GFP to the DM sheet induces forced fiber differentiation. Fibers that join the medial part of the myotome containing preexisting pioneers are properly patterned (delimited by white lines, in I the control left side is presented for comparison). In contrast, MyoD-containing DM-derived fibers that localize to the lateral myotome are highly disorganized as revealed by GFP staining (I, J) and general desmin immunoreactivity (K). Abbreviations, NT, neural tube.

white lines), and a lateral region devoid of medial pioneers in which the MyoD-induced fibers from the DM were disorganized similar to what we observed upon lateral somite electroporation. This result suggests that the relative organization of myotomal fibers depends upon interactions with medial pioneers.

Medial pioneers pattern differentiation of lateral myoblasts as well as of fibers originating from the extreme DM lips

To directly test the hypothesis that medial pioneers pattern the lateral myotome, a dominant-negative version of MyoD was electroporated into medial epithelial somites, that express MyoD from epithelial stages onward (Kahane et al., 1998b). This mutant construct consists of the bHLH domain of mouse MyoD fused to the engrailed repressor domain (MyoD(bHLH)-enR) (Steinbach et al., 1998). First, to control for the efficiency of MyoD(bHLH)-enR, we co-transfected this plasmid together with MyoD into the DM sheet, which mainly produces mitotic myoblasts and dermis (Ben-Yair and Kalcheim, 2005). Whereas MyoD/GFP alone stimulated extensive ectopic myofiber differentiation at the expense of mitotic myoblasts and dermis ($N=20$, Supplementary Figs. 2A–C, see also Figs. 6I–K, and not shown), co-transfection with MyoD(bHLH)-enR totally prevented myofiber generation ($N=15$, Supplementary Figs. 2D–F). As control, we used a very similar plasmid in which the basic region was replaced by the corresponding sequence of the

MyoD's non-myogenic dimerization partner E12 (E12 basic (bHLH)-enR). This construct precludes inactivation of MyoD targets, and in our in vivo assay it was indeed ineffective in preventing fiber development upon MyoD treatment ($N=10$, data not shown). Taken together, we confirmed previous findings demonstrating the efficiency of MyoD(bHLH)-enR as an inhibitor of myogenesis (Steinbach et al., 1998) to be used in our system.

Next, we overexpressed MyoD(bHLH)-enR/GFP into medial pioneers and reincubated the embryos for 33 h, a time when a significant amount of lateral fibers is expected to be present. A stronger electroporation protocol was used for these functional assays when compared to that used for lineage tracing, as this enabled to transfect a larger amount of pioneer cells (see Materials and methods and compare Figs. 7B, E with Fig. 1C). Dominant-negative MyoD prevented pioneer myoblasts from differentiating into myofibers; instead, GFP-labeled pioneer cells lacked desmin immunoreactivity, remained aggregated and relocated mainly to the sclerotome thus resulting in a smaller and abnormal pioneer myotome (Figs. 7A, B and D, $N=37/62$). Consequently, the lateral portion of the myotome composed of ELMs was abnormally patterned as revealed both by fewer and

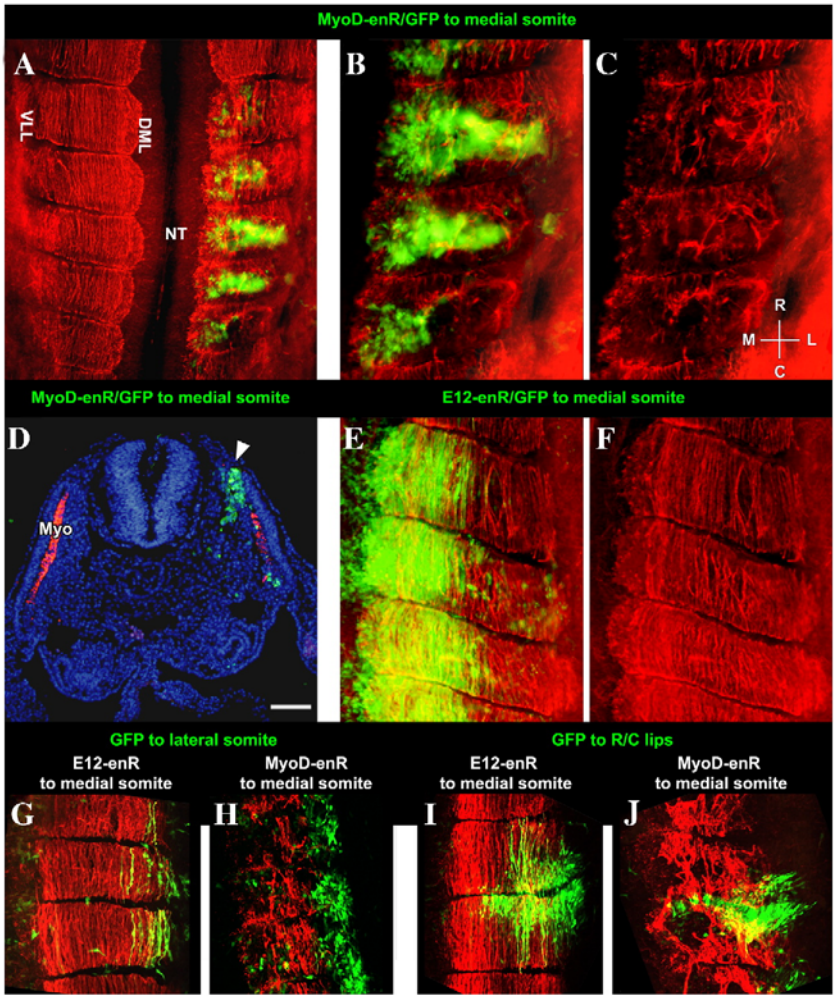

Fig. 7. Inhibiting development of medial pioneers by dominant-negative MyoD severely affects differentiation of cells emanating from the lateral somite and from rostral and caudal DM lips. (A–D) Electroporation of MyoD(bHLH)-enR/GFP (MyoD-enR) to the medial aspect of somites. Panels A–C are whole-mount preparations with panels B and C showing a higher magnification of the treated side in panel A. Note in panels A and B that GFP-labeled cells failed to form myofibers and remained aggregated while the entire mediolateral extent of the myotome shows aberrant fiber alignment as revealed by desmin expression (red). (D) Transverse section showing that GFP-labeled medial progenitors failed to incorporate into the myotome and remain aggregated (arrowhead); the desmin+ myotome is significantly smaller in the treated when compared to contralateral side. (E, F) Electroporation of E12 basic (bHLH)-enR/GFP (E12-enR) to the medial aspect of somites. Whole-mount preparation showing normal development of the pioneer myotome with fibers coexpressing GFP and desmin immunoreactivities (F, desmin staining, E, merge of desmin+ GFP). (G, H) Electroporation of unlabeled E12 basic (bHLH)-enR (G) or unlabeled MyoD(bHLH)-enR (H) to the medial aspect of somites and of control GFP to the lateral aspect. Note in panel G the normal development of both medial fibers that received E12 basic (bHLH)-enR as well that of lateral fibers labeled with GFP. In contrast, most of the myotome is mispatterned in panel H with GFP-labeled ELMs remaining mostly mesenchymal and desmin-negative. (I, J) Electroporation of unlabeled E12 basic (bHLH)-enR (I) or unlabeled MyoD(bHLH)-enR (J) to the medial aspect of somites and of control GFP to the extreme DM lips. Note in panel I the normal development of both medial fibers that received E12 basic (bHLH)-enR as well that of fibers labeled with GFP that elongate from both rostral and caudal lips of the DM toward the opposite pole of each segment. In contrast, most of the myotome is mispatterned in panel J with GFP-labeled cells from the extreme lips remaining either in the epithelium or forming severely misaligned fibers. The scheme in panel C depicts orientations (R, rostral, C, caudal, M, medial, L, lateral) applicable to all panels. Abbreviations, DML, dorsomedial lip, Myo, myotome, VLL, ventrolateral lip. Scale bar=60 μM.

misaligned desmin-positive fibers (Figs. 7A–C). In fact, the overall organization of the myotome was severely affected suggesting that pioneer fibers are required for patterning the entire myotome including ELMs. In contrast, similar over-expression of E12 basic (bHLH)-enR/GFP into medial pioneers was compatible with normal formation of both the medial as well as the lateral myotomal domains ($N=60/60$, Figs. 7E, F).

To further validate the above results, we directly visualized the formation of fibers stemming from the lateral somite as well as from the extreme rostral and caudal DM lips. First, E12 basic (bHLH)-enR or MyoD(bHLH)-enR (without GFP) was similarly delivered to the medial somite and GFP-DNA separately transfected into cells of the lateral somite. As expected, normally organized GFP+ lateral fibers (as well as GFP−/desmin+ medial fibers) were present in segments that received E12 basic (bHLH)-enR medially ($N=12$, Fig. 7G). In striking contrast, MyoD(bHLH)-enR that prevented differentiation of the medial pioneers yielded GFP+ ELMs that remained either epithelial or mesenchymal and when differentiated, were abnormally patterned ($N=12$, Fig. 7H). Altogether, these results demonstrate that correct differentiation of medial pioneers is required for proper organization of the early lateral myotome. Consistent with this notion, a more significant phenotype was frequently observed in younger rather than older flank-level somites emphasizing the need for an early inhibition of MyoD target genes in order to maximally reduce the number of pioneer fibers generated (not shown). Hence, even if ELMs do not intercalate among medial pioneers, their correct differentiation and organization depend upon signals emanating from these medial fibers, in direct continuity to which the ELMs develop.

Next, we asked whether the observed patterning activity of medial pioneers also operates on second wave fibers originating from the rostral and caudal lips of the DM, that intercalate among the pioneers (Kahane et al., 1998a, 2002). As shown in Figs. 7I and J, transfection of the medial somite with MyoD (bHLH)-enR (without GFP) followed by electroporation of rostral or caudal somite lips with GFP-DNA resulted in the formation of few and abnormally aligned labeled fibers ($N=11$, Fig. 7J). In contrast, a similar treatment with E12 basic (bHLH)-enR had no appreciable effect on medial pioneers and fibers stemming from the extreme lips normally developed ($N=11$, Fig. 7I). Altogether, these results show for the first time that medial pioneers are required for proper organization of the myotomal fibers originating from diverse epithelial sources.

Discussion

We characterize the initial phases of development of myogenic progenitors that originate in the lateral domain of the epithelial somite at flank levels of the axis and that, upon differentiation, complement the medial pioneers in the formation of the young myotome. Our results show that while delamination and localization of these ELMs occur soon after somite dissociation, acquisition of MyoD followed by differentiation into myofibers is delayed in comparison to the medial pioneers. This delay is accounted for by the inhibitory activity of LPM-derived BMP which maintains them in a progenitor state likely until arrival of medial pioneer fibers. Most importantly, we demonstrate that ELM patterning is induced by the medial pioneers which also act as a scaffold for proper organization of additional DM lip-derived myofibers. Medial pioneers are, therefore, a unique population of myogenic progenitors endowed with self-organizing capacity that patterns the entire myotome.

Hence, in the flank, medial pioneers play a double role, they pattern the medial (epaxial) myotome arising from the extreme DM lips (this study) whose fibers elongate while intercalating among preexisting pioneers (Cinnamon et al., 1999; Kahane et al., 1998a, 2002), and also directly organize the earliest component of the lateral (hypaxial) myotome which differentiates mediolaterally in direct continuity with the medial pioneers rather than intercalating among its fibers. Since electroporation is a mosaic technique, we developed conditions to transfect a significant population of progenitors without disrupting the overall organization of somites. Under these conditions, we noticed that lateral fiber alignment was disrupted. In some instances, when few medial pioneers were attained, the lateral myotome seemed intact, suggesting that there is a threshold of pioneer differentiation required for correct patterning of lateral fibers. Furthermore, when the medial fibers were significantly affected, ELMs still delaminated but remained desmin-negative and mesenchymal, suggesting that medial pioneers not only affect lateral fiber assembly but also their actual differentiation. Since electroporation, even if massive, never attains the entire population, the possibility remained that the chaotic organization of fibers upon MyoD gain or loss of function results from miscommunication between adjacent transfected and untransfected cells rather than from the absence of a pioneer scaffold. To control for this possibility, we electroporated MyoD in a similar mosaic manner into the overlying DM sheet, which does not normally express MyoD nor does it generate fibers. Under these conditions, the induced fibers translocated into the underlying myotome. In the latter we distinguished two regions, a medial region where the MyoD+ DM-derived fibers met the medial pioneers and gave rise to ordered parallel fibers even if transfection was of the mosaic type, and a lateral region devoid of medial pioneers in which the MyoD-induced fibers from the DM were disorganized. Thus, we conclude that the relative organization/disorganization of fibers depends upon interactions with medial pioneers rather than from interactions among neighboring fibers that received or did not receive MyoD or MyoD-enR.

In zebrafish, the elongation of fast muscles was shown to be triggered by mediolaterally migrating slow muscle cells and in this case, initiation of the myogenic program was found to be separable from fiber elongation (Henry and Amacher, 2004). In spite of some similarities, it remains as yet unknown whether there is any homology between avian medial pioneers and zebrafish slow myoblasts (Kalcheim et al., 1999). In the zebrafish as well as in the avian myotome, the molecular signals that mediate lateral fiber patterning remain to be elucidated and may include factor/s secreted by the medial pioneers and/or cell contact-dependent mechanisms (Bajanca et al., 2006; Cinnamon et al., 2006; Cortes et al., 2003; Henry and Amacher, 2004).

Along this line, we found that although N-cadherin-mediated adhesion is necessary for maintaining the epithelial conformation of the DM, neither gain nor loss-of-N-cadherin function in the medial somite had an effect on pioneer fiber assembly (Cinnamon et al., 2006) in contrast to the reported effect of N- and M-cadherin on migration of zebrafish slow fibers (Cortes et al., 2003). ELMs were similarly refractory to N-cadherin misexpression (Kahane and Kalcheim, unpublished data), suggesting that mechanisms other than N-cadherin-mediated adhesion operate to pattern the early avian myotome. Interestingly, additional interactions between medial and lateral mesoderm domains were documented already at segmental plate levels, as the lateral half of the plate failed to segment if isolated from its medial counterpart (Freitas et al., 2001).

We show that medial pioneers pattern fiber alignment in the epaxial region. Since the ELM-derived fibers are the first lateral fibers to differentiate, we hypothesize that they may similarly organize DM lip-derived fibers in the hypaxial domain of the flank myotome. Consistent with this notion, previous pulse-chase experiments with tritiated thymidine revealed an early population of post-mitotic cells not only in epaxial but also in hypaxial muscles where new fibers were intercalated among the early founders (Kahane et al., 2002). While we originally inferred that the entire population of early post-mitotic cells (epaxial and hypaxial) arises from the medial somite (Kahane et al., 2002) which contains the earliest progenitors that withdraw from the cell cycle (Kahane et al., 1998b), our new data stemming both from Brdu analysis as well as GFP lineage tracing for 2 days, reveal that in the flank, the post-mitotic cells originally observed in the pulse-chase experiments within the hypaxial myotome likely derived from the lateral somite. Thus, we now reinterpret our data to support a separation of epaxial and hypaxial lineages from the first stages of myogenesis onward (Ahmed et al., 2006; Cheng et al., 2004; Ordahl and Le Douarin, 1992). In this regard, the ELMs may serve as a template that participates, by fusing with younger cells, in the formation of the observed multinucleated fibers within intercostal muscles (Kahane et al., 2002).

At early stages of somitogenesis, LPM-derived BMP4 establishes the mediolateral polarity of the somite by antagonistically controlling transcription of *Sim1* and *MyoD*, respectively (Capdevila and Johnson, 1998; Hirsinger et al., 1997; Marcelle et al., 1997; Pourquie et al., 1996; Reshef et al., 1998; Tonegawa and Takahashi, 1998). Following initial mediolateral specification, we find that BMP4 prevents premature myogenic differentiation by inhibiting the onset of MyoD transcription in ELMs that already delaminated and localized to their final position but, nevertheless, remain in a mesenchymal state. Consistent with the need for MyoD activation for fibers to differentiate, overexpression of noggin in the lateral somite accelerated the timing of MyoD transcription, desmin appearance and lateral fiber generation. Notably, the fibers that formed upon exposure to noggin preceded the arrival of the medial pioneers, and similar to ELMs whose differentiation was force-induced by ectopic MyoD, they were in many cases inappropriately patterned. Together with direct inhibition of the medial pioneers, these two lines of evidence strengthen the view that medial pioneers pattern the lateral myotome and further suggest that one function of BMP is to delay ELM differentiation until the arrival of the medial myotome. Hence, the time-dependent downregulation of BMP4 in the LPM is likely to be coordinated with the differentiating mediolateral wave of medial pioneer fibers in order to ensure the formation of a coherent tri-dimensional structure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.02.030.

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